Involvement of osteopontin and its signaling molecule CD44 in clinicopathological features of Adult T cell Leukemia

博士論文

Involvement of osteopontin and its signaling molecule CD44 in clinicopathological features of Adult T cell Leukemia

（成人 T 細胞白血病におけるオステオポンチンとその刺激伝達分子 CD44 の臨床病理学的意義について）

東北大学大学院 医学系研究科 医科学専攻

内科病態学講座　感染病態学分野

浩日勒

Contents

1. Abstract

Background: It was previously reported that the osteopontin (OPN) gene, as well as CD44, is trans-activated by the Tax protein of HTLV-1. However the synthesis of both in Adult T cell Leukemia (ATL) has not been described yet. Here I demonstrate the expression of these molecules in the plasma and tissue of ATL.

Method and Materials: OPN and soluble CD44 (sCD44) in plasma were measured by ELISA in four subtypes of 27 ATL patients and 30 normal individuals. The expressions of OPN, CD44 and CD44v6 in tissues were also studied by immunohistochemical staining in 7 ATL patients. Further, we investigated the expression of OPN and its receptor in two ATL cell lines using 43Tb(-) and ED40515(-).

Result: Elevated levels of the plasma OPN and sCD44 were found in patients with the ATL. The median levels of the plasma OPN were apparently higher in the acute type (843ng/ml, range: 438-2965ng/ml) compared with lymphoma (800ng/ml, range: 471-1023ng/ml), chronic (318ng/ml, range: 122-716ng/ml) or smoldering (259ng/ml, 196-321ng/ml) types of ATL. The median results of the plasma levels of sCD44 were as follows: acute type (441ng/ml, range: 68-897ng/ml), lymphoma type (257ng/ml, range: 135-524ng/ml), chronic type (125ng/ml, range: 94-395ng/ml), and smoldering

(193ng/ml, range: 174-212ng/ml). Significant differences were found among the normal and four subtypes in both OPN ($p=3.6x10^{-6}$) and sCD44 ($p<0.001$). These two markers were significantly related to each other ($p<0.002$) and were significantly associated with the performance status, total number of involved lesions, and lactic dehydrogenase, and inversely with lymphocyte count ($p<0.01$). Immunohistochemical analysis of lymph nodes and skin using anti-OPN and anti-CD44 antibodies showed that both were weakly or moderately positive in ATL cells but moderately or strongly positive in infiltrated macrophages in 6 patients, but the anti-CD44v6 antibody staining were only weakly positive in ATL cells in 2 patients. Further FACS analyses showed that the expression of integrin α4 in the 43Tb(-) ATL cell line was inhibited by the recombinant-OPN.

Conclusion: These results demonstrate that OPN and CD44 play important roles in disease prognosis and their products in plasma could be markers of the severity in ATL.

2. Introduction

Adult T cell leukemia (ATL) was the first human cancer found to be caused by a retrovirus, human T cell lymphotropic virus type-1 (HTLV-1) [1, 2]. Its transmission takes place through blood transfusion, mother's milk, and sexual intercourse. Its clinical entity was established in Japan [3], and the discovery of HTLV-1 enabled the molecular diagnosis of ATL, which has been classified into acute, chronic, lymphoma and smoldering types reflecting their diverse clinical features and prognosis [4]. The prognosis of acute and lymphoma-type ATL patients is extremely poor despite the introduction of intensive chemotherapy and allogeneic hematopoietic transplantation [5].

The clinical manifestation of ATL is usually characterized by leukocytosis, lymphadenopathy, hepatosplenomegaly, skin eruption, thrombocytopenia and hypercalcemia [3-5]. Most ATL cells are mature helper T cell phenotypes $CD3^+$, $CD4^+$, $CD8^-$, and $CD25^+$, with some exceptions such as double negative or double positive CD4 and CD8 [6, 7]. The pathological findings of ATL cells are 1) diffuse proliferation of the neoplastic cells, 2) pleomorphism of the neoplastic cells with markedly deformed nuclei, 3) heterogeneous histological features of lymph nodes admixed with a cluster of non-malignant lymphocytes, proliferation of

macrophages and well developed high endothelium venules and 4) high incidence of skin lesions due to the infiltration of neoplastic cells [8].

HTLV-1 infection is usually asymptomatic, but it can cause ATL after a long latency period. A study performed in Nagasaki, Japan showed that the lifetime risk to develop ATL among 100,000 HTLV-1 infected carriers aged 30 or older was approximately 6.6% (95%CI 3.8-9.2) for men and 2.1% (95%CI 1.0-3.1) for women [9]. Although the mechanisms of such leukaemogenesis are still not clear, numerous studies have been done to clarify how cancer develops from HTLV-1. HTLV-1 does not have an oncogene, but does have a unique gene Tax which activates long-terminal-repeat (LTR) in Trans [10]. Accordingly, a number of reports showed that cytokines and chemokines such as IL-1, IL-13, and IL-9 are synthesized by ATL cells [11-13]. But it is also true that tax expression is very low or undetectable in primary ATL cells [14].

OPN is a secreted glycoprotein that interacts both with CD44 and integrin including $\alpha 4\beta 1$, $\alpha 4\beta 7$, $\alpha 9\beta 1$ [15, 16]. OPN promotes the migration and invasion of tumor cells, inhibits apoptosis, and facilitates extracellular matrix remolding and angiogenesis by interacting with its receptors on various types of cells [17]. OPN is widely distributed in the body and secreted by a wide range of cells such as macrophages, activated T cells, endothelial cells, and vascular muscle cells [15]. It

is also a pro-inflammatory marker in various infectious diseases [18]. In a recent study, circulating OPN was described as the only cytokine elevated in mice bearing instigating tumors as opposed to non-instigating tumors [19]. CD44 is a broadly distributed transmembrane glycoprotein originally identified as a receptor for hyaluronan [20], and OPN was also proposed to be a ligand for CD44 [21]. It is synthesized in multiple isoforms that consists of the smallest CD44, which is known as standard CD44, and several larger variant isoforms, CD44v1-10 [22]. In clinical studies of patients with cancer, enhanced CD44 of the standard form as well as its variants forms, especially CD44v6, was correlated with poor prognosis [23, 24]. Dr. Zhang have reported that the OPN gene as well as CD44 is trans-activated by the Tax protein of HTLV-1 via PI3K/AKT and noncanonical nuclear factor (NF-kB) signaling pathway, respectively [25, 26]. However, the synthesis of both OPN and CD44 in ATL patients has not been described yet. Therefore, I measured the levels of OPN and soluble CD44 (sCD44) in the plasma of ATL patients to explore their relationship with various clinical prognostic factors. In addition, I examined whether these molecules are expressed in ATL cells in the involved lymph nodes and skin of patients using immune-staining and also in ATL cell line in vitro by FACS, and discussed the clinico-pathological roles of these molecules in ATL.

3. Objectives

To investigate the expression of OPN and CD44 in the plasma and involved tissue of ATL patients.

To see whether those molecules are correlated with disease severity in ATL.

4. Materials and methods

Plasma: Heparin plasma samples of 27 ATL patients were collected from Nagasaki University Hospital, in which 13 were acute, 3 were lymphoma, 9 were chronic and 2 were the smoldering subtypes (Table1). These patients were clinically diagnosed as ATL based on the accepted criteria for clinical, hematological and laboratory findings during the years 1991-2006. Samples were collected before treatment. Plasma was obtained by centrifugation at 3,000 rpm for 10 minutes at 4^{o}C and was aliquoted to cryotubes and stored at -80^{o}C until use. Multiple thawing was avoided. All work was conducted in accordance with the Declaration of Helsinki. This study was approved by the Ethics Committees of Tohoku University Hospital (2007-257) and Nagasaki University Hospital (14090570). In addition, plasmas from 30 healthy individuals (28±8 yr, 10M/20F) were included as the normal control.

Clinical data: The performance status (PS) (0/1/2, 3/4), total involved lesions (TIL) (1/2-3/>=4, 5), lactic dehydrogenase (LDH), lymphocyte (Ly) (%), white blood cell numbers (WBC), platelet count (PLT) and other laboratory markers were measured in Nagasaki University Hospital (Table2).

Enzyme-linked immuno sorbent assay (ELISA): OPN in the plasma and in the supernatant of the cell lines was measured using a human osteopontin Elisa kit (Immuno-Biological Laboratories, Gunma, Japan) as described previously [18, 27]. Plasma sCD44 was determined by Human CD44 Elisa Kit (Abcam, Japan) [28]. All measurements were based on the average of triplicate samples. Both the OPN and sCD44 levels in plasma were expressed as ng/ml. The normal ranges of plasma OPN and sCD44 were defined as mean±2SD of normal control group (Table2). High levels of plasma OPN/sCD44 were defined as 2 times of mean+2SD (Figure2).

Histological analysis: Tissues from lymph-nodes and skin from 7 ATL patients were analyzed. This study was approved by the Tohoku University Ethics Committee (2007-413). The tissues were fixed in 10% formalin and histological specimens for pathological diagnosis and then the immunohistochemical staining were treated according to usual methods as described previously [29]. I examined

the expression of OPN and its receptor CD44, and CD44v6 because CD44v6 is known to be induced in tumor cells by the interaction of OPN with its receptor, CD44 and/or integrin [30]. CD68, a marker of macrophages, was also used.

For OPN staining, antigen retrieval was performed in an autoclave (120^{o}C, 5 min) and anti-OPN monoclonal antibody (mAb) (1:250 dilution, MPIIIB10$_1$, DSHB) was used as the primary antibody [31] with a signal amplification staining system (CSA Ⅱ, Dako). For CD68, CD44 and CD44v6 staining, antigen retrieval was done by trypsin treatment (0.05%) for 30 min at 37 ^{o}C, and anti-CD68 (1:200 dilution, clone: PG-M1, DAKO), anti-CD44 (1:2000 dilution, clone: 2C5, R&D) or anti-CD44v6 (1:250 dilution, clone: VFF-7, Abcam) mAbs were used.

The expression levels were evaluated according to the proportion and intensity of the granulation on the tumor area; the intensity of the staining was recorded as follows: -, negative; +/-, weakly positive; +, moderately positive; ++, strongly positive.

Cells: 43Tb(-) and ED40515(-) of ATL cell lines were kindly provided by Dr. Maeda from Kyoto University, and were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 u/ml penicillin and streptomycin. Both cell lines are known to be derived from a leukemic clone. 43Tb(-) was

established from a patient with gastrointestinal involvement [32]. In addition, a macrophage cell line, A-THP-1, from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University) was used in the same condition as described above [33]. The supernatant of the cells was collected after four days culture of 1.5-2.0x10^6 in 10 ml of culture.

Flow cytometric analysis: 43Tb(-) and ED40515(-) cells were first grown in FBS free RPMI medium for 24 hours at 37◦C with 5% CO_2 and cultured with or without 3 μg recombinant OPN (R&D, Minneapolis) or the medium containing 10% supernatant of A-THP-1, a macrophage cell line. After 30 min incubation at 4◦C, cells were harvested and stained with mouse anti-human mAbs against CD44, CD44v6 (Abcam), CD49d (integrin α4), CD29 (integrin β1), integrin β7 (Biolegend), CD25 (BD Biosciences) and HLA-DR (BD Biosciences). All the mAbs were labeled with FITC or PE, and the stained cells were analyzed using a FACS Calibur Cytometer (BD Biosciences).

Statistical analysis: The Kruskal-Wallis test was used for estimating differences in the OPN levels among normal, acute, lymphoma, chronic and smoldering subtypes. Kendall rank correlation test were used for determining the relationship

between the clinical data and plasma levels of OPN and sCD44. In addition, Kaplan-Meier survival analysis with log-rank significance test was used to estimate the statistical difference in the survival rates between high and low OPN/sCD44 groups.

5. Results

Elevated levels of plasma OPN and sCD44 in ATL patients:

Plasma OPN and sCD44 were measured in 27 cases of ATL patients and 30 normal control individuals. The median levels of the plasma OPN were apparently higher in the acute type (843ng/ml, range: 438-2965ng/ml) compared with lymphoma (800ng/ml, range: 471-1023ng/ml), chronic (318ng/ml, range: 122-716ng/ml), or smoldering (259ng/ml, 196-321ng/ml) types of ATL (Table2). The levels were significantly different among normal and ATL subtypes and among the four subtypes, but not among the subtypes when excluding acute ATL according to the two tail kruskal-Wallis test ($p=3.6x10^{-6}$, $p<0.01$, $p=0.1$ respectively) (Figure 1A). It is of note that plasma levels of OPN were higher than the normal range (>396ng/ml) was found in all of the 13 acute and 3 lymphoma type patients and in 2 of the chronic type.

The levels of plasma sCD44 in ATL were also measured. The results were as follows: acute type (441ng/ml, range: 68-897ng/ml), lymphoma type (257ng/ml, range: 135-524ng/ml), chronic type (125ng/ml, range: 94-395ng/ml), and smoldering (193ng/ml, range: 174-212ng/ml) (Table2). The results of statistical analyses were essentially similar to those of OPN and were significantly different among the groups according to the two tail Kruskal-Wallis test (Figure 1B). However, differing from the levels of OPN, the levels of sCD44 lower than the normal levels (<194ng/ml) were found in 2 acute patients and 1 lymphoma patient.

Further, the relationships between the levels of OPN and sCD44 were assessed by two tail Kendall rank correlation test and the result showed a significant correlation between them in the ATL patients (n=27, τ (Kendall's tau coefficient)=0.44, $p<0.002$) (Figure 1C), but not in the normal individuals (n=30, p=0.1).

Plasma levels of OPN and sCD44 correlated with disease severity in ATL:

At present, several major prognostic indicators for ATL have been described, such as advanced PS, more than three TIL, high LDH, hypercalcemia and older than 40 years [34]. The correlations between the levels of plasma OPN and sCD44 with the above clinical data were estimated by two tail Kendall rank test. The

plasma OPN levels were found to have significant positive correlations with PS ($p<0.001$), TIL ($p<0.01$) and LDH ($p<0.05$) and an inverse correlation with Ly ($p<0.05$). The plasma sCD44 levels were also positively correlated with PS ($p<0.01$), TIL ($p<0.05$), LDH ($p<0.001$), and with soluble interleukin-2 receptor (sIL-2R) ($p<0.05$) and WBC ($p<0.05$); an inverse correlation was found with PLT ($p<0.01$) and Ly ($p<0.001$) (Table3).

Furthermore, Kaplan-Meier survival analysis with log-rank significance test was used to estimate the statistical difference in the survival rates between the high and low groups. High levels of plasma OPN/sCD44 were defined as 2 times of mean+2SD.The result showed that high levels of plasma OPN as well as sCD44 predicted low survival in ATL patients, as shown in Figure 2 ($p=0.009$, $p=0.03$ respectively). Other soluble markers of ATL, LDH and sIL-2R, were also analyzed and the survival rate in sIL-2R was not significantly different between the high and low groups (Figure 2).

OPN was synthesized not only by ATL cells but also by other cells:

To define the synthesizing cells in tissue, the expression of OPN, CD44, and CD44v6 were examined using tissues from lymph nodes and skin from ATL patients. CD68 was also used because macrophage cells are known to be OPN

producers. The tissues were infiltrated with diffusely proliferating tumor cells with markedly deformed nuclei, macrophages and normal lymphocytes [8].

Immuno-histological analysis showed that the infiltrated macrophages, which were identified by anti-CD68 mAb expressed OPN moderately or strongly in all cases but the infiltration of macrophages was also variable as only a few macrophage cells were seen in patient 2 (Table4) (Figure 3B, G). Furthermore, tumor cells were also positive for OPN staining in the tissues of ATL (Table4) (Figure 3C, H); however, the staining intensities were not so strong despite the amplification (Table 4). The cells of epidermis abundantly expressed OPN and some of the infiltrated ATL cells also expressed OPN (patient 2) (Figure 3C). It is possible that OPN synthesized by epidermal cells attract ATL cells to skin. In nasal cavity-associated lymphoid tissue (NALT) (patient 3), OPN was expressed by macrophage cells as well as by some ATL cells (Figure 3G, H).

CD44 staining was observed in tumor cells of 6 of the 7 ATL patients and in macrophage cells in all but patient 2 (Table4). But compared to OPN staining, CD44 staining was apparently more evident in ATL tumor cells (Figure 3D, I). For CD44v6 staining, positive tumor cell staining was only weakly positive in patients 5 and 6 (Table4); Figure 3E&J, the cells in epidermis were positive for CD44v6, but the tumor cells and macrophage cells in NALT were negative.

The expression of integrin and CD44 in the ATL cell lines:

Further, to investigate the interaction and relationship between OPN and its receptor integrin and CD44, I utilized 43Tb(-) and ED40515(-) cells ATL cell lines in vitro. First, I examined the OPN production of 43Tb(-) and ED40515(-) cells and found that the ATL cell line itself produces lower amounts of OPN in vitro compared with the macrophage cell line of A-THP-1 cells. The levels of OPN in the supernatants in the 43Tb(-), ED40515(-) and A-THP-1 cell lines were 200pg/ml, 400pg/ml and 43ng/ml, respectively. Then, the expressions of integrin α4, β1, β7, and CD44 and CD44v6 were measured with the positive controls of CD25 and HLA-DR in 43Tb(-) and ED40515(-) cells. FACS analysis showed that both cell lines were positive for α4 integrin, CD25 and HLA-DR, but negative for integrin β1, β7 and CD44, CD44v6 (Table5).

Therefore, we could not detect interaction between OPN and CD44 or CD44v6 in the above ATL cell lines, but could show that the α4 integrin expression of 43Tb(-) was inhibited by both rOPN and the supernatant OPN from A-THP-1, whereas those of CD25 and HLA-DR were not inhibited (Figure 4A-C).

6. Discussion

In this study, the clinicopathological roles of both OPN and sCD44 in ATL were studied. The results showed that the plasma levels of both molecules were elevated in ATL patients and the levels were significantly different among normal and the four ATL subtypes. Furthermore, the plasma levels of two molecules were significantly correlated and both levels were clinically associated with the prognostic factors of ATL of PS, TIL, LDH and Ly. However, the level of OPN had strong correlations with clinical markers such as PS and TIL, whereas that of sCD44 was correlated strongly with laboratory markers such as Ly and LDH. In addition, plasma levels of sCD44 were also significantly associated with sIL-2R indicating that the levels directly reflect the tumor burden. The correlation of OPN with TIL was explained in part by its chemoattractive activity [35], but these distinctly different correlations between OPN and sCD44 have never been described in other human cancers.

I next examined the expression of CD44 and OPN in ATL tissues. The source of OPN has been believed to be tumor cells [36] and /or other cells such as macrophages [37]. In this study, OPN was only detected after the amplification and ATL cells synthesized OPN and/or CD44 weakly or moderately. Dr. Zhang reported that Tax enhanced the expression of OPN and CD44 [25, 26], however, it is

also known that leukemic cells lack the expression of virus-related genes including Tax in ATL cells. The correlation of the expression of these molecules with that of Tax should be carefully examined in future *in vivo.* In normal lymph nodes, OPN staining was positive in B cells of Germinal Center (GC), endothelial cells and macrophages (data not shown). In the 6 ATL patients, infiltrated macrophage cells were positive for both OPN and CD44 and their intensities were much stronger than those of ATL tumor cells. Also, previous report showed that cells from lymph nodes express more activated antigens such as CD38, Ki-67, CD7 and HLA-DR than peripheral blood leukemic cells in ATL, and proposed that ATL cells proliferate mainly in the lymph nodes [38]. Intracytoplasmic staining of OPN was demonstrated in OPN secreting fetal rat calvarial cells [39]. Therefore, probably the increased plasma levels of OPN in ATL are derived from tumor-associated (infiltrated) macrophages and tumor cells in the enlarged lymph nodes of ATL. It is quite interesting that, macrophages in the involved tissues of the ATL lymph nodes were strongly positive for both OPN and CD44. Tumor-associated macrophages are known to be the M2 phenotype that promotes tumor proliferation [40]. Further, recent research revealed that increased numbers of CD68-positive tumor-associated macrophages in lymph nodes were strongly associated with treatment failure, relapses and shortened survival in patients of classic Hodgkin's

lymphoma [41]. Although the signalling of OPN through CD44 was implicated to be important in breast cancers and tumour-associated macrophages were reported to synthesize OPN and CD44 in an experimental mouse model [42], this is the first report that both molecules were found in macrophages and tumor cells in human diseases. Therefore, plasma levels of OPN may be a good prognostic marker because it could reflect the activities of tumor-associated macrophages. Moreover, although a number of cytokines were known to be released from HTLV-1 infected cell lines or fresh tumour cells, none of them were found to be correlated with the prognosis [11-13]. However, CD44v6 expression in infiltrating ATL cells was found only in two cases and it was unexpectedly strongly expressed in the epidermis. In addition, abundant expression of OPN in epidermal cells was noted, which may be the cause of the frequent skin involvement in ATL because OPN is also known as a chemoatrractant agent [35]. Also, it would be of interest to determine if the cells of the epidermis in ATL patients synthesize more OPN than that of normal individuals. Additionally, two ATL cell lines, 43Tb(-) and ED40515(-), were negative for both CD44 and CD44v6, but positive for integrin α4. Although, we could not detect integrin β1 and β7 on these cell lines, the presence of functional integrins were strongly suggested and further analysis of integrins on

these cell lines are on the way to investigate the chemo-attractive activity [15, 35] of OPN on ATL cells.

7. Conclusions

Consequently, this is the first report describing both OPN and CD44 expression in ATL. Because OPN-CD44 axis was found to be associated with the prognosis of ATL patients, these molecules could be potential targets in this aggressive disease. However, since the numbers of patients studied here was small, it will be necessary to investigate the roles of OPN and CD44 in a larger number of patients. It would also be important to determine whether these molecules could be markers to detect the progression of the disease in HTLV-1 infected carriers.

Taken together, this study clarified that elevated levels of plasma OPN and sCD44 were associated with the disease severity in ATL patients and that they were expressed in lymph-node and skin. Due to their multiple biological activities, both could be prognostic markers of ATL.

8. Acknowledgement

First, I would like to express my thanks to the people who assisted me in this study: Dr. K. Tsukasaki from Nagasaki University and Professor H. Harigae from Tohoku University Hospital for supplying the patient samples and clinical data; to Dr. Y. Takahashi for her help in immunohistochemical staining and to Professor M. Fukumoto for kind guidance in interpreting the slides for immunohistochemical staining; to Professor N. Ishii for kind advice on FACS analysis; to Dr. J. Zhang from Osaka FUSO Pharmaceutical Industries for her discussion on the molecular mechanism of Tax and to Dr. S. Oguma from Kyoto Takeda General Hospital for great help in statistical analysis; also to Dr. M. Maeda from Kyoto University for providing the 43Tb(-) and ED40515(-) cells and to Dr. A. Kasajima from Tohoku University Hospital for her kind advice on interpreting the slides of immunohistochemical staining. .

Second, I am grateful to Dr.s Y. Ashino, H. Saitoh and E. Kodama for their great help in my research at Tohoku University and also to my colleagues S. Li, XG. Li, U. Ruman Siddiqi and B. Shiratori for their t help in every step of my study and for encouragement each other.

Finally, I would like to express my deep and sincere gratitude to Professor Toshio Hattori, his great help and encouragement enabled me to finish the present

thesis.

In addition, I would like to thanks to the following Professors and Doctors who gave important comments on current thesis: Professor M. Satake from Division of Molecular Immunology, Institute of Development, Aging and Cancer, Professor N. Tanaka from Department of Cancer Medical Science, Dr. K. Kikuchi from Department of Dermatology and Dr. T. Takahashi from Department of Microbiology and Immunology.

This work was supported by collaborative funding from the Research Center for Zoonosis Control, Hokkaido University.

9. Reference

1) Poiesz BJ, Ruscetti FW, Gazdar AF, et al: Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc Natl Acad Sci USA 1980; 77(12): 7415-7419.

2) Hinuma Y, Nagata K, Hanaoka M, et al: Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. Proc Natl Acad Sci USA 1981; 78(10):6476-6480.

3) Uchiyama T, Yodoi J, Sagawa K, et al: Adult T-cell leukemia: clinical and hematologic features of 16 cases. Blood 1977; 50: 481-492.

4) Shimoyama M: Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma: A report from the Lymphoma Study Group (1984- 1987). Br J Haematol 1991; 79:428-437.

5) Tsukasaki K, Hermine O, Bazarbachi A, et al: Definition, prognostic factors, treatment, and response criteria of adult T-cell leukemia-lymphoma: a proposal from an international consensus meeting. J Clin Oncol 2008; 27:453-459.

6) Hattori T, Uchiyama T, Toibana T, et al: Surface phenotype of Japanese adult T-cell leukemia cells characterized by monoclonal antibodies. Blood 1981; 58 (3):645-647.

7) Suzushima H, Asou N, Nishimura S et al: Double-negative (CD4- CD8-) T cells

from adult T-cell leukemia patients also have poor expression of the T-cell receptor alpha beta/CD3 complex. Blood 1993; 81(4):1032-1039.

8) Kikuchi M, Mitsui T, Matsui N, et al: T-cell malignancies in adults: histopathological studies of lymph nodes in 110 patients. Jpn J Clin Oncol 1979; 9: 407-422.

9) Arisawa K, Soda M, Endo S, et al: Evaluation of adult T-cell leukemia/lymphoma incidence and its impact on non-Hodgkin lymphoma incidence in southwestern Japan. Int J Cancer 2000; 85(3):319-324.

10) Grassmann R, Dengler C, Müller-Fleckenstein I, et al: Transformation to continuous growth of primary human T lymphocytes by human T-cell leukemia virus type I X-region genes transduced by a Herpesvirus saimiri vector. Proc Natl Acad Sci USA 1989; 86:3351-3355.

11) Wano Y, Hattori T, Matsuoka M, et al: Interleukin 1 gene expression in adult T-cell leukemia. J Clin Invest 1987; 80:911–916.

12) Chung HK, Young HA, Goon PK, et al: Activation of interleukin-13 expression in T cells from HTLV-I-infected individuals and in chronically infected cell lines. Blood 2003; 102:4130–4136.

13) Chen J, Petrus M, Bryant BR, et al: Induction of the IL-9 gene by HTLV-I Tax stimulates the spontaneous proliferation of primary adult T-cell leukemia cells by a paracrine mechanism. Blood 2008; 111(10):5163-5172.

14) Satou Y, Yasunaga J, Yoshida M, et al: HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells. Proc Natl Acad Sci USA 2006; 103(3):720-725.

15) Katagiri YU, Sleeman J, Fujii H, et al: CD44 variants but not CD44s cooperate with beta1-containing integrins to permit cells to bind to osteopontin independently of arginine-glycine-aspartic acid, thereby stimulating cell motility and chemotaxis. Cancer Res 1999, 59(1):219-226.

16) Green PM, Ludbrook SB, Miller DD, et al: Structural elements of the osteopontin SVVYGLR motif important for the interaction with alpha(4) integrins. FEBS Lett 2001; 503(1):75-79.

17) Bellahcène A, Castronovo V, Ogbureke KU, et al: Small integrin-binding ligand N-linked glycoproteins (SIBLINGs): multifunctional proteins in cancer. Nat Rev Cancer 2008; 8(3):212-226.

18) Chagan-Yasutan H, Saitoh H, Ashino Y, et al: Persistent elevation of plasma osteopontin levels in HIV patients despite highly active antiretroviral therapy. Tohoku J Exp Med 2009; 218: 285-292.

19) McAllister SS, Gifford AM, Greiner AL, et al: Systemic endocrine instigation of indolent tumor growth requires osteopontin. Cell 2008; 133(6):994-1005.

20) Aruffo A, Stamenkovic I, Melnick M, et al: CD44 is the principal cell surface

receptor for hyaluronate. Cell 1990; 61(7):1303-1313.

21) Weber GF, Ashkar S, Glimcher MJ, et al: Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science 1996; 271(5248):509-512.

22) Ponta H, Sherman L, Herrlich PA: CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 2003; 4(1):33-45.

23) Stauder R, Eisterer W, Thaler J, et al: CD44 variant isoforms in non-Hodgkin's lymphoma: a new independent prognostic factor. Blood 1995; 85(10):2885-2899.

24) Wielenga VJ, van der Neut R, Offerhaus GJ, et al: CD44 glycoproteins in colorectal cancer: expression, function, and prognostic value. Adv Cancer Res 2000; 77:169-187.

25) Zhang J, Yamada O, Matsushita Y, et al: Transactivation of human osteopontin promoter by human T-cell leukemia virus type 1-encoded Tax protein. Leuk Res. 2009; 34:763-768.

26) Zhang J, Yamada O, Kida S, et al: Identification of CD44 as a downstream target of noncanonical NF-kB pathway activated by Human T-cell leukemia virus type 1-encoded Tax protein. Virology 2011; 413:244-252.

27) Chagan-Yasutan H, Shiratori B, Siddiqi UR, et al: The increase of plasma galectin-9 in a patient with insulin allergy: a case report. Clin Mol Allergy 2010; 8(1):12.

28) Chen R, Sigdel TK, Li L, et al: Differentially Expressed RNA from Public Microarray Data Identifies Serum Protein Biomarkers for Cross-Organ Transplant Rejection and Other Conditions. PLoS Comput Biol 2010; 6(9): e1000940.

29) Shibata H, Yamakoshi H, Sato A, et al: Newly synthesized curcumin analog has improved potential to prevent colorectal carcinogenesis in vivo. Cancer Sci 2009; 100(5):956-60

30) Gao C, Guo H, Downey L, et al: Osteopontin-dependent CD44v6 expression and cell adhesion in HepG2 cells. Carcinogenesis 2003; 24(12):1871-1878.

31) Takemoto M, Yokote K, Nishimura M, et al: Enhanced expression of osteopontin in human diabetic artery and analysis of its functional role in accelerated atherogenesis**.** Arterioscler Thromb Vasc Biol 2000; 20(3):624-628.

32) Hattori T, Asou N, Suzushima H, et al. Leukaemia of novel gastrointestinal T-lymphocyte population infected with HTLV-I. Lancet 1991; 337(8733):76-77.

33) Tominaga T, Suzuki M, Saeki H, et al: Establishment of an activated macrophage cell line, A-THP-1, and its properties. Tohoku J Exp Med 1998; 186(2):99-119.

34) Major prognostic factors of patients with adult T-cell leukemia-lymphoma: A cooperative study—Lymphoma Study Group (1984-1987). Leuk Res 1991; 15:81-90.

35) Weiss JM, Renkl AC, Maier CS, et al: Osteopontin is involved in the initiation of cutaneous contact hypersensitivity by inducing Langerhans and dendritic cell migration to lymph nodes.J Exp Med 2001; 194(9):1219-1229.

36) Castellano G, Malaponte G, Mazzarino MC, et al: Activation of the osteopontin/matrix metalloproteinase-9 pathway correlates with prostate cancer progression. Clin Cancer Res 2008; 14(22):7470-7480.

37) Hsu HP, Shan YS, Lai MD, et al: Osteopontin-positive infiltrating tumor-associated macrophages in bulky ampullary cancer predict survival. Cancer Biol Ther. 2010; 10(2):144-154.

38) Shirono K, Hattori T, Hata H, et al: Profiles of expression of activated cell antigen on peripheral blood and lymph node cells from different clinical stages of adult T-cell leukemia. Blood 1989; 73:1664-1671.

39) Zohar R, Lee W, Arora P, et al: Single cell analysis of intracellular osteopontin in osteogenic cultures of fetal rat calvarial cells. J Cell Physiol 1997; 170(1):88-100.

40) Solinas G, Germano G, Mantovani A, et al: Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation.J Leukoc Biol 2009; 86(5):1065-1073.

41) Steidl C, Lee T, Shah SP, et al: Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. N Engl J Med 2010; 362(10):875-885.

42) Duff MD, Mestre J, Maddali S, et al: Analysis of gene expression in the tumor-associated macrophage. J Surg Res 2007; 142(1):119-128.

10. Figure legends

Figure1.The plasma levels of osteopontin and sCD44 in normal and four subtypes of ATL and their relationships.

Plasma OPN and sCD44 were measured in 27 cases of ATL patients and 30 normal control individuals. (A) OPN. The median levels of the plasma OPN were apparently higher in the acute type (843ng/ml, range: 438-2965ng/ml) compared with the lymphoma (800ng/ml, range: 471-1023ng/ml), chronic (318ng/ml, range: 122-716ng/ml), or smoldering (259ng/ml, 196-321ng/ml) types of ATL. The levels were significantly different among normal and ATL subtypes and among the four subtypes, but not among subtypes when excluding acute ATL according to the two tail kruskal-Wallis test ($p=3.6x10^{-6}$, $p<0.01$, not significant (NS) respectively). (B) sCD44. The levels of plasma sCD44 in ATL were as follows: acute type (441ng/ml, range: 68-897ng/ml), lymphoma type (257ng/ml, range: 135-524ng/ml), chronic type (125ng/ml, range: 94-395ng/ml), and smoldering (193ng/ml, range: 174-212ng/ml). The results of the statistical analyses were essentially similar to those of OPN and were significantly different among groups according to the two tail kruskal-Wallis test ($p<0.001$, $p<0.05$, NS respectively). (C) The relationships between the levels of OPN and sCD44 were assessed by two tail kendall rank correlation test and the result

showed a significant correlation between them in ATL patients (n=27, τ (kendall's tau coefficient) =0.44, $p<0.002$).

Figure2. Plasma OPN levels predicts survival rates of ATL.

Kaplan-Meier survival analysis with log-rank significance test was used to estimate statistical differences in the survival rate between high and low groups. The results showed that high OPN (>792 ng/ml) and sCD44 (>392 ng/ml) levels predicted low survival rates ($p<0.01$, $p<0.05$ respectively). Other prognostic soluble markers, LDH (500U/L) and sIL-2R (10000 U/ml) were also analyzed and sIL-2R was found not to be significant in the survival rate between high and low groups.

Figure3. Immunohistochemical stainings of CD68, OPN, CD44 and CD44v6 in ATL Immunostaining of skin (A-E, patient 2) and nasal cavity-associated lymphoid tissue (NALT) (F-J, patient 3) are shown.

All pictures were x40 magnifications. (A) Hematoxylin and eosin (HE) staining showed tumor cells and normal epidermis cells in skin. (B) Few infiltrating CD68 positive macrophages were found. (C) Some infiltrated ATL cells expressed OPN and the cells of the epidermis were strongly positive for OPN. (D) Most tumor cells as well as normal epidermis cells were positive for CD44. (E) The cells in the epidermis but not

the ATL cells were positive for CD44v6. (F) HE staining showed tumor cells with markedly deformed nuclei. (G) Infiltrating CD68-positive macrophages are shown. (H) Infiltrated macrophages strongly expressed OPN. (I) Tumor cells and macrophage cells were positive for CD44. (J)Tumor cells from the ATL lacked the expression of the CD44v6.

Figure4. Recombinant OPN (rOPN) inhibits the binding of antibody against integrin α4 but not those of CD25 and HLA-DR in 43Tb(-) cells.

43Tb(-) cells were cultured with FBS free RPMI for 24 h at 37◦C with 5% CO_2 and were treated 30 min with 3μg/ml of rOPN or A-THP-1 supernatant at 4◦C followed by FACS analysis after washing. Both rOPN and A-THP-1 supernatant inhibited the binding of antibody against integrin α4 (A), but not that of CD25 (B) or HLA-DR (C). The color of each line: black, isotype; green, control; pink: rOPN; blue: A-THP-1 supernatant.

11. Figures

Figure1.

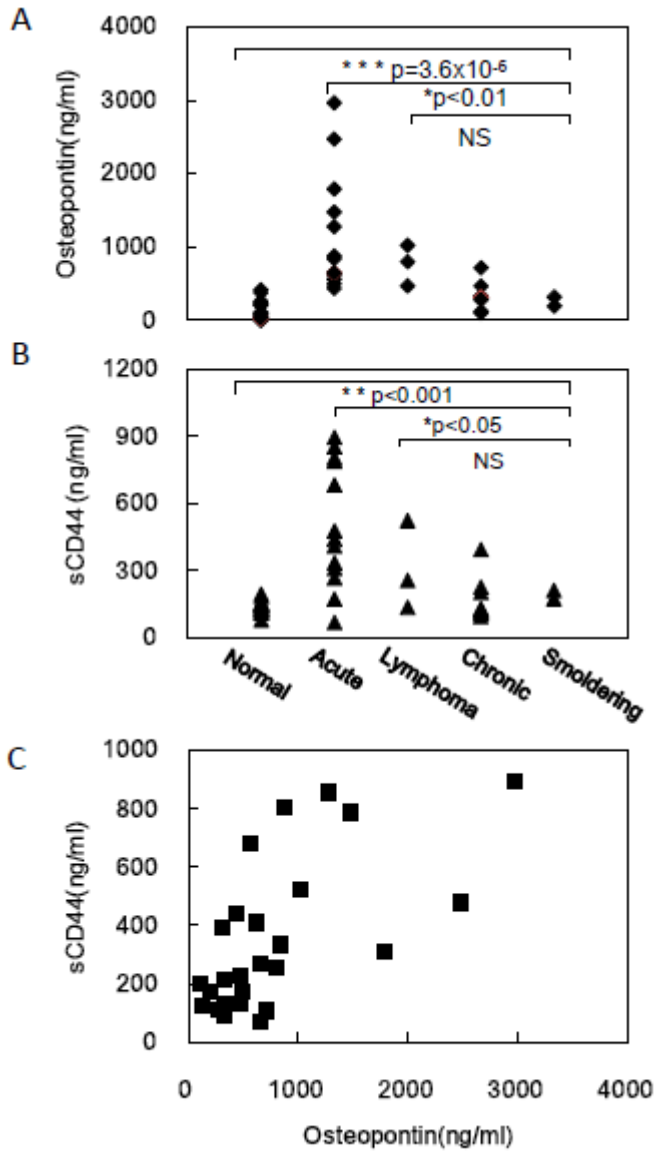


Figure2.

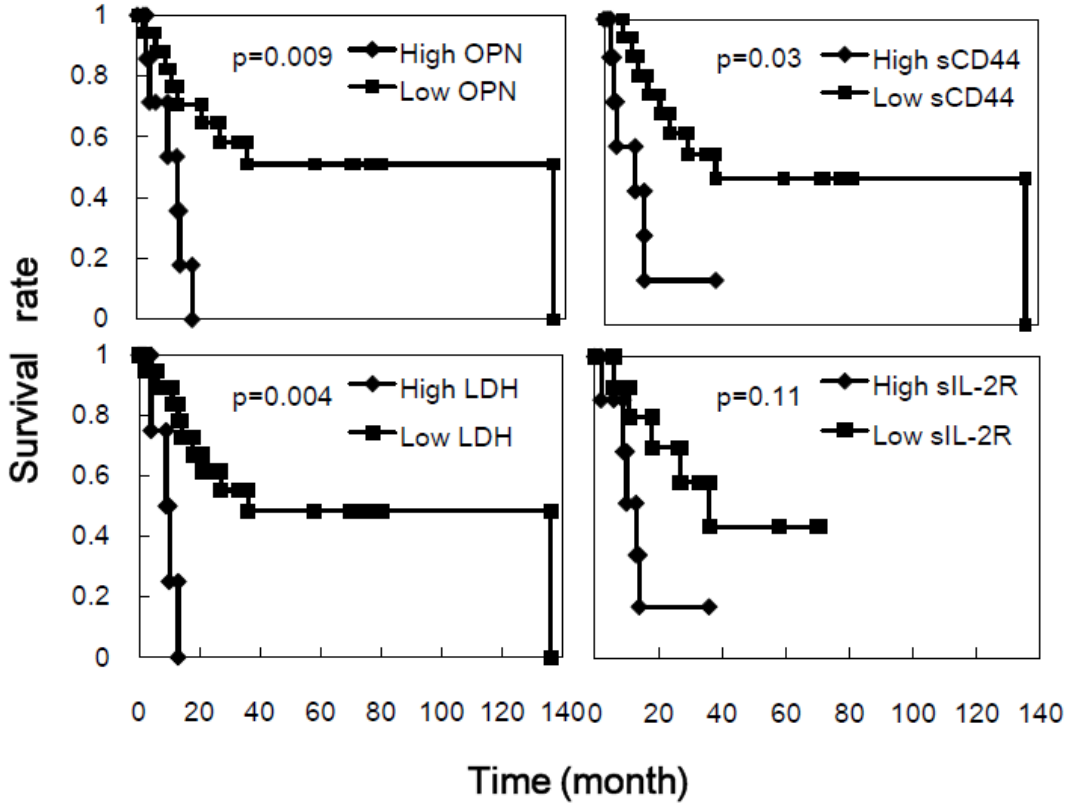

Survival rate
1
0.8
0.6
0.4
0.2
0
p=0.009
High OPN
Low OPN
p=0.03
High sCD44
Low sCD44
p=0.004
High LDH
Low LDH
p=0.11
High sIL-2R
Low sIL-2R
0 20 40 60 80 100 120 1400 20 40 60 80 100 120 140
Time (month)

Figure3.

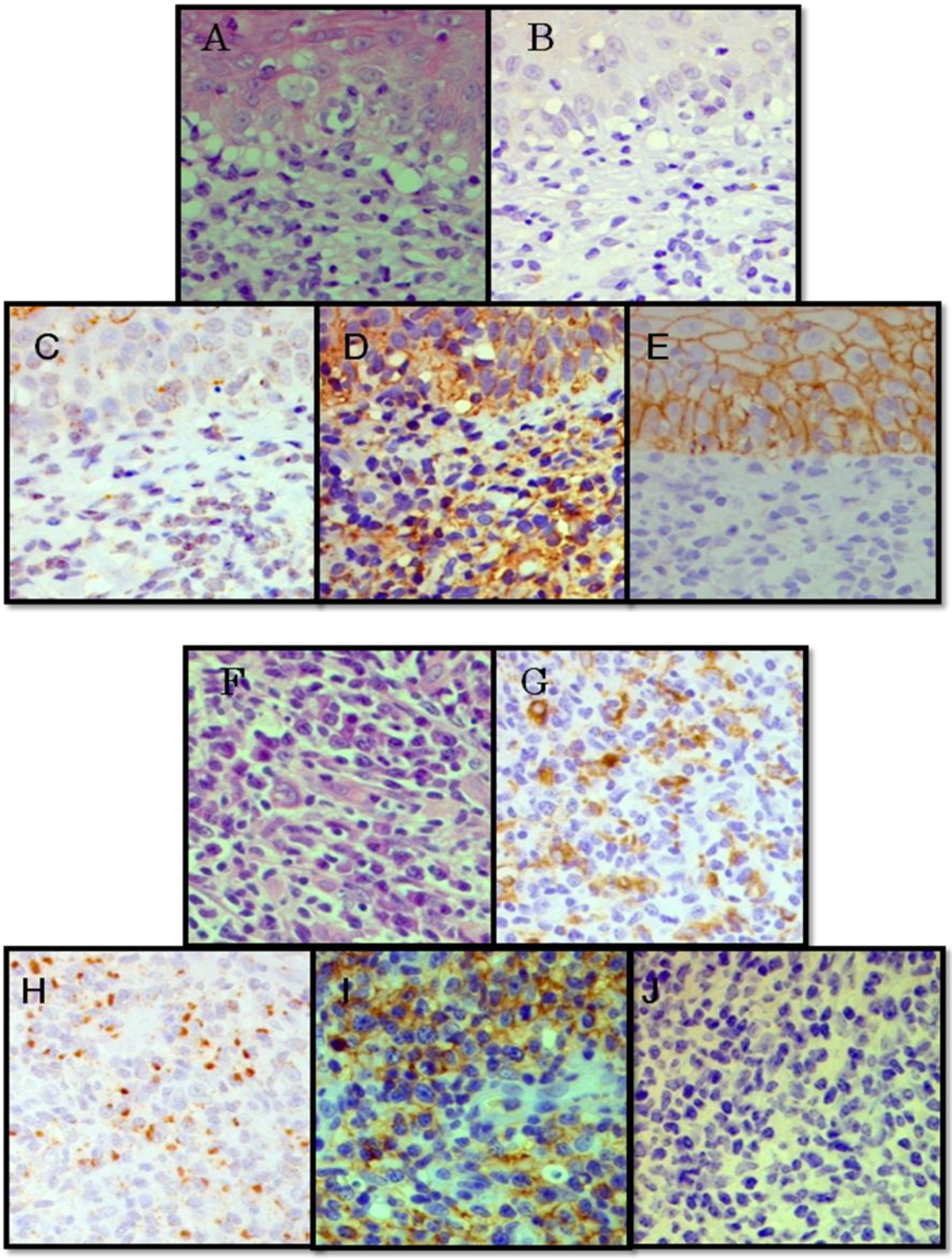
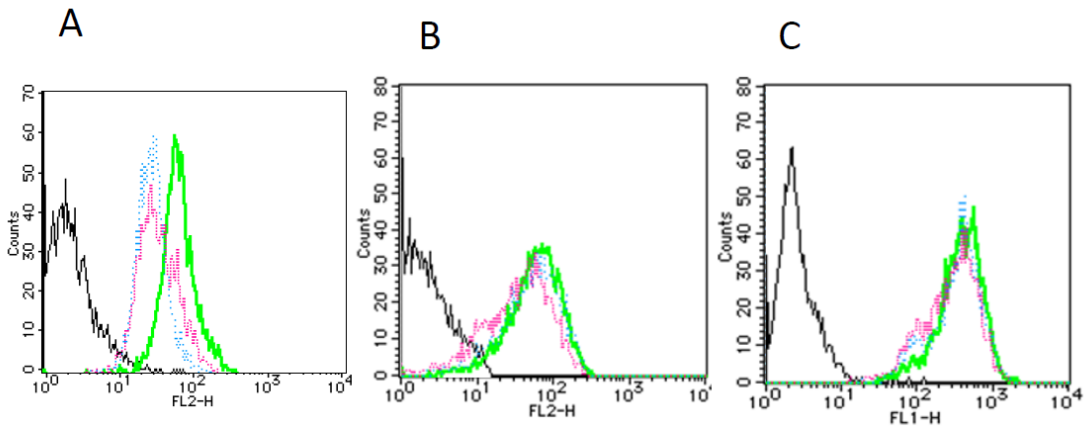


Figure4.



12. Tables

Table1. Basic Characteristics of ATL study group

		Acute	Lymphoma	Chronic	Smoldering	Total
	Subject no.	13	3	9	2	27
Age	(mean±SD) (yr)	60 ±13.6	65±14.5	56±16.2	66±6.4	60±14
Sex	(male / female)	10M/3F	2M/1F	4M/5F	1M/1F	17M/10F

Abbreviations: F, female; M, male.

Table2. Clinical characteristics of ATL patients.

Characteristics	Ref. range	Acute	Lymphoma	Chronic	Smoldering
		Median (range)			
WBC- 10^3/uL	3.2-9.6	34(7.4-203)	3.2(3-7.8)	12.9(5.3-22.7)	6.8(6.3-7.2)
HB - g/dL	13-17	13.6(11.7-17.4)	11(10.4-12.6)	12.1(11.5-15.8)	12.8 (11.4-14.2)
PLT - 10^3/uL	131-362	173(98-399)	172(135-185)	249(152-334)	215 (214-216)
Ly - %	22-55	6(0-18)	30(26-45)	31(14-58)	30.5 (29-32)
Abnormal Ly - %	0	51(6-96)	1(0-1)	11(5-70)	19.5 (7-32)
CRP - mg/dL	0-0.3	0.9(0.23-3.8)	0.9(0.23-1.5)	0.2(0.05-3)	0.1 (0.03-0.1)
PS - 0/1/2,3/4	0	2(1-4)	1(1-1)	1(0-1)	0 (0-0)
TIL - 1/2-3/>=4,5	0	4(2-5)	2(1-2)	1(1-3)	1(1-1)
LDH - U/L	119-229	1075 (150-9165)	502 (203-639)	217 (155-361)	193 (187-199)
sIL2R - U/ml	145-519	108084 (1902-127703)	8084 (2266-61388)	1530 (798-12575)	1412 (543-2280)
Hypercalcemia- Y/N	N	8Y/5N	N	N	N
OPN - ng/ml	0-396	843(438-2965)	800(471-1023)	318(122-716)	259 (196-321)
CD44 – ng/ml	83-194	441(68-897)	257(135-524)	125(94-395)	193(174-212)

Abbreviations: ATL, Adult T cell Leukemia; WBC, white blood cell; HB, hemoglobin; PLT, platelet counts; Ly, lymphocyte; CRP, C-reactive protein; PS, performance status; TIL, total involved lesions; LDH, lactic dehydrogenase; sIL-2R, soluble interleukin 2 receptor; OPN, osteopontin; sCD44, soluble CD44; Y, yes; N, no.

Table3. The relationship between clinical characteristics and plasma levels of OPN & sCD44 of ATL patients.

Characteristics	OPN(ng/ml)		sCD44(ng/ml)	
	τ	p	τ	p
WBC (10^3/mL)	0.13	0.32	0.28	0.04 *
HB (g/dL)	0.09	0.50	-0.008	0.5
PLT (10^3/mL)	-0.15	0.27	-0.43	0.002 **
Ly (%)	-0.34	0.01 *	-0.5	0.0001 ***
Abnormal Ly (%)	0.15	0.26	0.2	0.09
CRP (mg/dL)	0.21	0.19	0.13	0.4
PS (0/1/2,3/4)	0.48	0.0006 ***	0.4	0.004 **
TIL (1/2-3/>=4,5)	0.37	0.008 **	0.3	0.03 *
LDH (U/L)	0.31	0.02 *	0.5	0.0002 ***
sIL-2R (U/mL)	0.31	0.07	0.52	0.03 *

Statistical analysis was made by Kendal-rank test. τ means kendall's tau coefficient; * $p<0.05$; ** or *** $p<0.01$.

Table4. Immunohistochemical findings of OPN & CD44, CD44v6 staining in tissue of patients with ATL

Patient No.	Age/Sex	Diagnosis	Sample type	IHC findings			Plasma OPN (ng/ml)
				OPN-CSA Ⅱ	CD44	CD44v6	
1	39F	ATL	Lymph-node	T-, M++ , E+	T+, M+	T-	970
2	54F	ATL	Skin	T+, Epi+	T+, Epi+	T-, Epi+	610
3	48M	ATL	Nasal Cavity	T+, M++	T+, M++	T-	3550
4	59F	ATL	Lymph-node	T+, M++, E+	T+, M++	T-,F+	NA
5	70F	ATL	Lymph-node	T+, M+	T+/-, M+	T+/-	NA
6	48M	ATL	Lymph-node	T+, M+	T-, M+, E+	T+/-	NA
7	36M	ATL	Lymph-node	T+/-, M+, E+	T+, M+	T-	NA

Abbreviations: IHC, immunohistochemical staining; F, female; M, male; ATL, Adult T cell Lymphoma; T, tumor cell; M, macrophage; E, endothelial cell; Epi, epithelial cell; F, fibroblast cell; NA, not available; -, negative; +/-, weakly positive; +, moderately positive; ++, strongly positive.

Table5. FACS analysis showed integrin α4 is positive in 43Tb(-) & ED40515(-) ATL cells.

Antibody	Clone	43Tb(-)	ED40515(-)
CD49d(integrin α4)	9F10	99%	95%
CD29(integrin β1)	TS2/16	N	N
Integrin β7	FIB504	N	N
CD44	F10-44-2	N	N
CD44v6	VFF-7	N	N
CD25	2A3	99%	95%
HLA-DR	G46-6	97%	91%

Abbreviation: N, negative.